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Award Number: DAMD17-00-1-0362

TITLE: The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

PRINCIPAL INVESTIGATOR: Janice L. Huff, Ph.D.

CONTRACTING ORGANIZATION: BioForce Nanosciences, Incorporated
Ames, Iowa 50010

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

5. FUNDING NUMBERS
DAMD17-00-1-0362

6. AUTHOR(S)
Janice L. Huff, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
BioForce Nanosciences, Incorporated
Ames, Iowa 50010

E-Mail:jhuff@bioforcenano.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This report details final work on this project that was performed while the principal investigator was at University of Nevada Las Vegas. This project has currently been re-defined and the work is being performed at BioForce Nanosciences, Inc. Ames IOWA. The goals of this project were to define signaling pathways triggered in immortalized breast epithelial cells (MCF10A) when these cells bind to and migrate on distinct forms of laminin-5. Activation of FAK and MAPK kinase were shown to occur upon engagement of non-migratory forms of laminin-5 only in the presence of the Beta 1 integrin activating antibody TS/216.

14. SUBJECT TERMS
cell signaling, cell migration, integrins, extracellular matrix

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction and Personal Statement:

This award has now been transferred to a new laboratory, BioForce Nanosciences in Ames Iowa. The new title is: Protein NanoArrays for Profiling Malignant Progression in Breast Cancer Cell Lines. The following report is the final summary of work performed under the previous title as described below. This work was carried out over the 18 month time period from June 30, 2000 to December 15, 2001. This work was abruptly interrupted when my old advisor moved to a new location. I submitted my yearly report in July of 2001, and my lab was being shut down by August of 2001. I remained until December of 2001 and tried to complete as much of the work as possible, before moving myself. Unfortunately, given the circumstances of the move and the fact that this project was fraught with multiple reagents problems, I was not able to finish much of the proposed work. The transfer of the award and the commencement of the new work plan began in October of 2002. I am including a copy of my new statement of work that describes what research I am currently addressing (successfully!).

Statement of Work

The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

Task 1. Analyze FAK activation and CAS phosphorylation following adhesion of MCF-10A's to pro-migratory, cleaved vs. uncleaved laminin-5 matrix.

a. Phosphorylation/activation state of endogenous FAK will be determined by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies and in vitro kinase assay following a time-course after adhesion to laminin-5.

Task 1 Progress Report (7/01): Preliminary experiments showed enhanced phosphorylation of both FAK and CAS following adhesion of MCF-10A's to cleaved, pro-migratory laminin-5. These experiments were carried out using immunoprecipitation with specific FAK or CAS antibodies followed by Western blotting with anti-phosphotyrosine antibodies. In vitro kinase assays to measure activation of FAK have not been completed to date.

Task 1 Progress Report (12/01). In vitro kinase assays were not performed.

Task 2. Analyze FAK activation and CAS phosphorylation following stimulation of MCF-10A's with β1-integrin activating antibody TS2/16.

Task 2 Progress Report: In preliminary experiments, I was unable to repeat the previously reported TS2/16-stimulated migration of MCF-10A's on laminin-5. To test if this was due to the batch of antibody that I was using I obtained the TS/216 hybridoma and prepared a fresh stock of the antibody from serum-free cell culture supernatants using a Protein G-Sepharose chromatography. The new stock of antibody has allowed me to repeat the

experiments showing enhanced migration of 10A's on laminin-5. Analysis of FAK and CAS phosphorylation have not been completed.

Task 2 Progress Report (12/01). ***Analysis of FAK and CAS phosphorylation were not performed using the new batch of TS/216.***

Task 3. Determine role of exogenously expressed FAK, CAS and respective mutants on migration of MCF-10A's.

a. Stable cell lines will be generated by transfection of MCF-10A's with expression vectors encoding epitope-tagged versions of FAK, CAS and variants.

Task 3a Progress Report (7/01): Three attempts were made in the past year to obtain stable MCF-10A cell lines over-expressing FAK, CAS and variants. FAK and CAS cDNA's were subcloned into pcDNA3.1 (Invitrogen), a eukaryotic expression vector containing a CMV-promotor and G418 drug resistance marker. In all attempts I was able to obtain numerous G418 resistant clones, but no clones over-expressed either FAK or CAS. The expression vectors were determined to be functional by transient transfection of COS cells. I am currently pursuing transient transfection experiments of MCF-10A cells using these vectors in combination with GFP as a marker for transfection efficiency.

b. Analyze migration of cell lines on laminin-5 and other extracellular matrix molecules.

c. Corroborate data generated from stable cell lines with analysis of same constructs in transient transfection assays.

Task 3a, b, c Progress Report (12/01): Transient transfection studies were not completed.

Task 4. Determine role of migration-linked signaling pathways in laminin-5 cleavage model.

a. Analyze cAMP levels in MCF-10's plated on cleaved vs. uncleaved laminin-5
b. Analyze activation of ERKs during migration on cleaved laminin-5

Task 4 Progress Report (7/01): Task 4 experiments have not been attempted to date.

Task 4 Progress Report (12/01): Studies assessing the activation of ERK upon engagement of MCF10 cells on Laminin-5, Fibronectin, Poly-L-Lysine were performed. Results showed that ERK became phosphorylated upon engagement with all of these substrates, only in the presence of the Beta1 integrin activating antibody TS/216.

Additions to original Statement of Work (7/01):

A) While performing experiments associated with Task 1 it became evident that much variation existed in stocks of cleaved laminin-5 that were produced by enzymatic digestion of purified matrix. To address this issue I am in the process of constructing a recombinant laminin-5 γ2 chain that is truncated at precisely the point of enzymatic cleavage. This construct contains a 6-His tag at the carboxyl terminus and a signal peptide at the amino terminus. A full-length γ2 chain construct with a 6-His tag is also being constructed. The goal is to express either the full-length or truncated molecule in either MCF-10A or the rat bladder carcinoma cell line 804G, which both express the laminin-5 β3 and α3 chains. Laminin-5 will be purified from cell culture supernatants using metal chelate affinity chromatography. Using this system I should be able to obtain laminin-5 preparations that only contain the cleaved or the uncleaved γ2 chain. Potential problems with this system are: 1) Improper or non-functional association of exogenously expressed truncated γ2 chain with endogenous α and β chains. However, evidence from the literature suggests that the exogenously expressed γ2 chain should associate normally with the endogenous α and β laminin chains. 2) Inability to purify sufficient quantities of recombinant laminin-5 using this system.

Progress Report on Addition 1 (12/01):

The His tagging and the fusion of a signal peptide sequence at the amino terminal cleavage site were performed. When I attempted to assemble these pieces into a full-length construct for testing I found multiple errors in the restriction enzyme map that I had been sent. Subsequent sequence analysis revealed that this cDNA was not what it was said to be. Ultimately, I ran out of time to finish this project.

B) Laminin-5 is not commercially available at this time. We have relied on an industry resource to obtain this material for our experiments. So I would not be dependent on this source alone, I have set up a system to purify laminin-5 from 804G cell culture supernatants using an antibody affinity column. (laminin-5 γ2 chain specific monoclonal antibody TR1). I have been able to isolate pure, soluble laminin-5 using this system and will use this as a back up if needed.

Progress Report on Addition 2 (12/01): Laminin-5 was purifed as described. However, I found that these cells not only secrete laminin-5, but they secrete a significant amount of fibronectin as well. This can be removed from the preparations by a preclearing step with a fibronectin antibody affinity column. This is an important factor in interpreting data from any studies done on matrix secreted by these cells and was something I was not aware of when I began this purification.

Key Accomplishments June 30, 2000 – July 31, 2001:

- Analysis of FAK and CAS phosphorylation following adhesion of MCF-10A's to promigratory, cleaved vs. uncleaved laminin-5.
- Determination that MCF-10A cells will not tolerate exogenously expressed FAK, CAS and variant molecules.
- Partial construction of a recombinant cleaved laminin-5 γ2 chain cDNA.
- **Publication of manuscript cited below.**

Key Accomplishments August 1, 2001 – December 15, 2001:

- Began analysis of ERK phosphorylation in MCF10A cells on laminin-5 in the presence of TS/216 antibody.
- Performed fluorescence analysis of MCF10A cells on Laminin-5.

Reportable Outcomes June 30, 2000 – July 31, 2001.

Publications and Abstracts:

Earley, B., Plopper, G.E. and J.L. Huff. The role of FAK in breast cell migration on laminin-5. Western Alliance of Medical Students Annual Meeting 2001.

Plopper, G.E., J.L. Huff, W.L. Rust, M.A. Schwartz and V. Quaranta. 2001. Antibody induced activation of β1 integrin receptors stimulates cAMP-dependent migration of breast cells on laminin-5. *Molecular and Cellular Biology Research Communications* **4**, 129-135.

Reportable Outcomes August 1, 2001 – December 15, 2001:

Publications: These projects were from graduate students that I helped train during the course of this award.

Wagner, J.E. , Huff, J.L., Rust W.L., Kingsley K., and Plopper, G.E. 2002. Perillyl alcohol inhibits breast cell migration without affecting cell adhesion. *J. Biomed. Biotechnol.* **2**, 136-14.

Kingsley, K., W.L. Rust, J.L. Huff, R.C. Smith and G.E. Plopper. 2002. PDGF-BB enhances expression of, and reduces adhesion to, laminin-5 in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **294,** 1017-1022.

Kingsley, K., J.L. Huff, W.L. Rust, K. Carroll, A. Martinez and G.E. Plopper. 2002. ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. *Biochem. Biophys. Res. Commun.* **293,** 1000-1006.

Patent Application: Method and System for In Vitro Screening of Compounds For Pro/Anti-Migratory Effects. Serial No. 60/183,628

Student Training (7/01):

Brian Earley, undergraduate Biology major, did an independent research project under my supervision from May 2000 to March 2001. He submitted an abstract on his project and presented a talk at the Western Alliance Medical Student Conference held in February in Carmel, CA. In addition, Brian was awarded an undergraduate research grant for $1000.00 for supplies from UNLV for work done on this project. Brian is currently applying for admission to medical school.

Kilpatrick Carroll, undergraduate Chemistry major, did independent research under my supervision during the first year of this grant. He worked extensively on the attempts to make MCF-10A cell lines. He is enrolled in graduate school at Columbia University for Fall 2001.

Michael Cascia, undergraduate Biology major, assisted in this project for a 3 month period (March 2001-May 2001). Michael utilized the laboratory research skills that he acquired to obtain employment as a laboratory technician at UCSF.

Conclusions: The analysis of integrin-mediated signaling that occurs in breast cells on distinct forms of laminin-5 was a project frought with numerous technical difficulties, and in addition, the laboratory where this work was being conducted was abruptly shut down when my advisor left for a new position in August of 2001. This award was transferred to BioForce Nanosciences, Inc. in October of 2002 and a new statement of work was approved for the duration of this award.
Technical issues tackled during this project: 1) Failure of integrin activating antibody to reproduce previously reported stimulation of migration. Freshly purified batch of this antibody was prepared and did show the enhanced migration effect, however time did not allow me to complete the research objectives described for use of this antibody.
2) Inability to obtain stable cell lines overexpressing FAK, CAS and variant proteins. Multiple attempts were conducted at obtaining stable cell lines transfected with constructs expressing these proteins, but only G418 resistant, non-expressing clones were obtained. This is a mystery to me still as the expression vectors worked well in a transient system and have worked in other laboratories for production of stable cell lines. It is possible that these constructs were toxic to the MCF10A cell line that I was using.
3) Laminin-5 cleavage products produced by enzymatic digestion of purified laminin-5 were not consistent and of high enough quality for reproducible experimental data on migration and signaling to be obtained. I embarked on the construction of a recombinant laminin-5 gamma-2 chain that was truncated at precisely the point of enzymatic cleavage. This cloning project progressed smoothly until it was revealed that the actual gamma-2 chain cDNA that I had obtained was incorrect. Ultimately, I ran out of time to finish this project.
I have attached the statement of work for the current research that I am conducting at BioForce Nanosciences for the duration of this award.

Revised Statement of Work for DAMD17-00-1-0362
Principal Investigator: Janice L. Huff, Ph.D.

I. Background:

Molecular medicine in the post-genomic era holds the promise of earlier cancer diagnosis and better chemotherapy choices by detecting and cataloging molecular signatures of pre-malignant disease and disease progression. This information will be useful for development of sensitive cancer screening tests, for monitoring clinical disease progression, for defining appropriate drug targets, and for monitoring individual patient's response to chemotherapeutic drugs [1,2,3].

To attain these goals the need exists for assay systems that can simultaneously measure multiple protein species with sensitivity to discriminate subtle quantitative as well as qualitative changes in these molecules and their binding partners. In addition, to be practical and clinically useful for large scale studies these assays must also meet the commercial requirements of speed, cost efficiency and requirement for minimal starting tissue material.

Practical microarray tests for protein-based screening are beginning to emerge. Current protein microarray assays have shown promise in meeting the needs for the type of assay described above [4,5]. In the most common format these assays involve mechanical deposition of suitable capture molecules, such as specific antibodies, in an array format onto an appropriate substrate. Mechanical deposition methods can be subdivided into two categories: controlled spray of microdroplets (i.e., inkjet printing) and pin tool deposition. Both of these approaches deposit nanoliter amounts of starting material, which correspond to molecular domain sizes ranging from slightly smaller than 100 μm^2 to 300μm^2. These arrays are readily multiplexed so that numerous protein targets can be studied in parallel. The reverse phase protein array is a variation on this assay format that was developed by investigators in the FDA-NCI clinical proteomics program to study changes in important cellular regulatory proteins during pre-malignant conversion within a single individual. In this assay format small quantities of protein lysates from laser dissected tissue biopsy samples (LCM) are directly deposited in an array format on nitrocellulose-covered glass slides. These slides are then interrogated with antibodies directed against proteins of interest [6,7]. This format allows direct comparison of changes in protein profiles of tiny, multiple tissue biopsy samples from a single individual and allows subtle changes in protein profiles that accompany pre-malignant conversion to be studied in detail. Although this system requires less starting tissue biopsy material than the more common antibody-capture format [8], it is still limited by the requirement of relatively large amounts of protein necessary for each successful test. *This is a key factor that negatively impacts the practical application of the current methodology for real world cancer biopsy screening where available sample size is vanishingly small.*

BioForce Laboratory, Inc., http://www.bioforcelab.com) is an industry leader in the development of next-generation biomolecular screening arrays, or NanoArrays, with patented technology permitting the construction of biochips with sub-micron spatial addresses. These nano-scale arrays allow a thousand or more molecular tests to be carried out in the same surface space occupied by a single state-of-the-art microarray spot. (See Figures 1 and 2) An obvious advantage of this design is the dramatic savings in reagent cost that accompanies ultra-miniaturization. *More importantly, it provides a realistic platform for performance of array-based analyses in applications involving extremely small quantities of sample material where uses of current microarray formats are not feasible.*

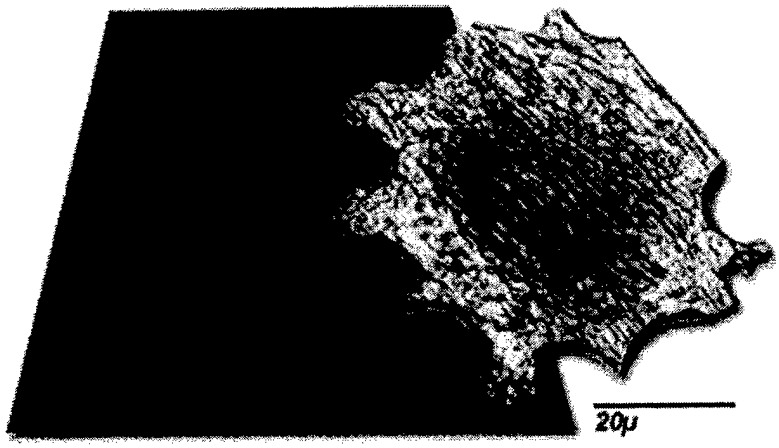
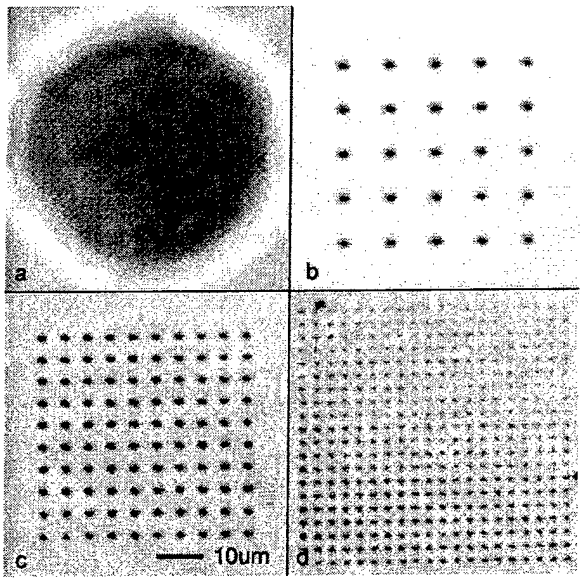


Figure 1: Size comparison of a NanoArray (~1 μm diameter spots at a density of 1 spot/ 4 μ^2, pitch = 3μ) with a typical frog retinal glial cell shown to scale.

Figure 2. Optical micrograph comparison of NanoArray and conventional microarray spot sizes. Panel a shows a 30μm diameter microarray spot generated by MicroJet deposition. Panels b, c and d show NanoArrays with 5 x 5, 10 x 10 and 20 x 20 spot densities, respectively.



The goal of this revised statement of work is to construct a nano-scale protein array platform and use it as a basic research tool to study alterations in cellular signaling pathways that accompany breast cancer disease progression. In the long term we anticipate that this assay will provide a much needed platform for breast cancer proteomics.

II. Specific Aims

Specific Aim 1: Determine precision and linearity of NanoArray platform using purified antigen.

1) A suitable protein, such as recombinant human estrogen receptor (#RDI-ERALPHA-AG Research Diagnostics Inc, NJ) will be arrayed in dilution curves on NanoArray chips and will be detected with an anti-estrogen receptor antibody (Research Diagnostics Inc., NJ), followed by a fluorescently labeled secondary antibody.

   From these control experiments we will determine: Intra- and Inter-spot reproducibility, limits of protein detection, and linearity of detection.

Specific Aim 2: Determine precision and linearity of NanoArray using protein lysates from breast cancer cell lines.

1) Defined cell lysates from standard breast epithelial cell lines representing various stages of clinical breast cancer progression (normal: MCF10A; MCF-7, MDA-MB-231 and MDA-MB-425) will be prepared for use in this phase of assay optimization.
2) Salt, protein and detergent concentrations may be altered to achieve optimal protein deposition.
3) Protein profiles of previously validated markers proteins will be generated during this phase of research. These markers will include Estrogen Receptor, Progesterone Receptor and HER2/*neu*.

Specific Aim 3: Study expression patterns of pro-survival markers in breast cancer progression cell line model.

1) Cell lysates will be prepared, arrayed and processed according to conditions established upon successful completion of Specific Aims 1 and 2.
2) Identically arrayed protein chips will be probed with monoclonal antibodies for pro-survival markers. Specifically, we will assess expression levels and phosphorylation states of Protein Kinase B/Akt and ERK, both protein kinases speculated to be important in regulation of cell proliferation and cell survival.
3) Protein loading will be normalized by measuring actin levels on identically arrayed chips.

III. Research Design and Methods:

A. Deposition substrate:

NanoArrays are constructed on a variety of debris-free and topographically flat substrates; and both chemically reactive and inert surfaces are available for testing.

1) Passive of molecules adsorption onto a gold coated glass or polished silicon wafer is the easiest and most straight forward method of protein deposition. This method works extremely well for arraying purified proteins under low salt conditions, and will be the first method tested for arraying of the cell lysates.

These substrates are prepared as follows: #1 glass cover slips (Fisher Scientific) or polished silicon wafers that were first cut into 4mm X 4mm squares are cleaned thoroughly in water followed by ethanol using an ultrasonic bath for 30 min. Following the cleaning, the substrates are stored in ethanol until used. Surfaces are coated with a thin layer (5 nm) of chromium at 0.1 nm/s followed by the deposition of 10 nm of gold at 0.2nm/ using an IBC 2000 ion-beam sputterer from South Bay Technologies, Inc. Substrates are masked before sputtering with a 200 mesh (lines/in) finder grid containing 105 μm x 90 μm holes. Figure 1 shows a schematic depiction of the layout of a NanoArray chip.

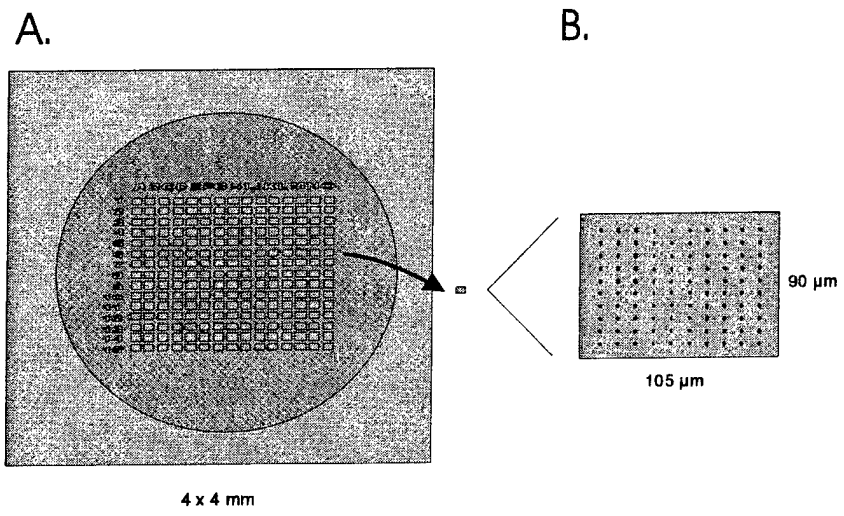


Figure 3. Schematic diagram of NanoArray chip. A. A 15 x 15 finder grid overlayed on a glass coverslip creates a mask which allows easy and accurate sample location on the NanoArray. B. Blow-up of a single 105 μm x 90 μm block showing a 10 x 10 NanoArray.

2) Covalently couple the proteins to a chemically activated surface. Several surface chemistry choices are available:

a. Self assembling monolayer (SAM) of succinimide terminated alkanethiolates on a gold surface. The succinimide groups react spontaneously with primary amines to form a covalent attachment between the surface and the biomolecules in the deposited sample.
b. Aldehyde activated surfaces are generated on mica or flat glass using APTES (aminopropyl triethoxysilane), followed by glutaraldehyde treatment.
c. Versalinx chemical affinity tools (Prolinx, Inc., WA). This is a synthetic, small molecule, low molecular weight affinity pair that is used for surface immobilization of a wide variety of macromolecules. Advantages include ease of use and high retention of ligand activity. Disadvantage, each sample needs to be linked to one member of the affinity pair.

3) Deposition onto hydrophilic polymers and gel-based matrices.

a. Hydrogel chips (Packard, CT) consist of a porous polyacrylamide matrix into which deposited proteins are immobilized. This provides an aqueous environment for subsequent antibody interactions.
b. FastSlides (Schleicher and Schuell, OR) are glass-backed nitrocellulose and are the substrate used in construction of reverse-phase protein microarrays.

Factors that will be considered when chosing an appropriate substrate are: preservation of biological activity of deposited molecules, protein binding capacity, and ease of use.

B. Deposition conditions:

1) NanoArrayer tip composition: We currently use a tip fabricated of silicon nitride for NanoArray deposition. This tip may be modified to increase protein loading and flow.
2) Room humidity: This is the relative humidity in the NanoArrayer housing, and is altered for optimal deposition depending on sample composition.

C. Post-deposition chip processing and optical detection:

1) Hydration: Time, post-deposition, in which the chip hydrates in a humid chamber beforeantibody incubation steps.
2) Standard optimization of antibody incubation steps.
3) Optical detection: Fluorochromes will be chosen to obtain the best signal intensity.

Figure 2 outlines steps in construction of a NanoArray with subsequent optical (fluorescent) readout.

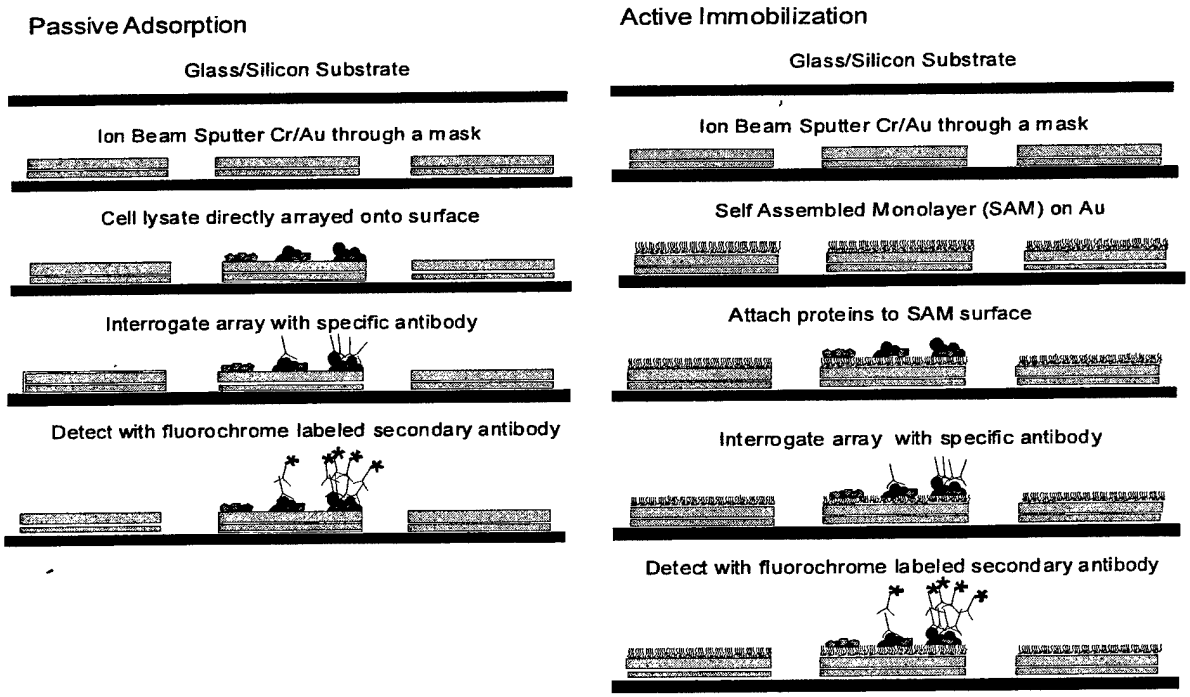


Figure 2. NanoArray construction and optical readout. NanoArrays can be constructed either by Passive adsorption (left side) or by an Active immobilization of the antibodies to the chip surface (right side). In both cases, either glass or silicon chips are first layered with chromium and then gold.

D. NanoArray Data Acquisition and Analysis:

1) Fluorescent image data will be taken from each array and stored in a tagged image file format (TIFF). The P-SCAN software package will be used for image analysis [7].
2) Data will be analyzed for reproducibility by depositing each sample in a minimum of 10 NanoArray spots.

3) The dynamic range of marker detection, or assay sensitivity in terms of cell numbers, will be assessed by analyzing data derived from dilution curves of the cellular lysates.

Project Timeline and Milestones:

<table>
<tr><th></th><th>Specific Aim 1</th><th>Specific Aim 2</th><th>Specific Aim 3</th></tr>
<tr><td rowspan="2">0-6 Months</td><td colspan="2">NanoArray Purified Proteins</td><td rowspan="3"></td></tr>
<tr><td>Deposition Conditions</td><td>Dynamic Range of Marker Detection</td></tr>
<tr><td rowspan="2">6-12Months</td><td rowspan="3"></td><td colspan="2">NanoArray Breast Cancer Cell Lines Known Markers</td></tr>
<tr><td>Deposition Conditions</td><td>Dynamic Range of Marker Detection</td></tr>
<tr><td>9-13 Months</td><td colspan="2"></td><td>NanoArray Breast Cancer Cell Lines Pro-Survival Markers</td></tr>
<tr><td>PROJECT MILESTONES</td><td>NanoArrays show reproducible and linear detection of recombinant Estrogen Receptor.</td><td>NanoArrays show reproducible and linear detection of Estrogen Receptor from human breast cell lines.</td><td>NanoArrays are useful for experimental data acquisition.</td></tr>
</table>

IV Literature Cited:

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Human Subjects Research: Claim of Exemption Form is attached.
Animal Use: This research does not involve the use of vertebrate animals.